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ELECTROPHORETIC CELL SEPARATION USING MICROSPHERES

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1. Introduction

Methods of cell separation based on the electrokinetic properties of the cell membrane offer a degree of discrimination among cell populations which is not available with methods based on cell size or density alone. Functionally homogeneous cell populations share a common surface topography in terms of various receptors, antigenic determinants, transport enzymes, etc., all of which possess numerous ionogenic groups which together define the net surface charge density of the cells at a given pH. Electrophoresis of a mixture of cell types should therefore allow separation into the constituent functional sub-populations, as a function of their differential cellular electrophoretic mobilities. In practice, numerous limitations apply to electrophoretic separation of cells. Free cell suspensions in isotonic media compatible with cell viability are mandatory. Maintenance of isotonicity by dissolved salts at physiological concentrations is not practical in electrophoretic terms, since the resulting high ionic strength of the medium compresses the electrokinetic double layer of the cells, decreasing their surface charge density and hence the electrophoretic mobility; in addition, current flow exceeds the capabilities of most electrophoresis power supplies. Media are therefore supplemented with sucrose, Ficoll, or dextrose, allows low ionic strength buffers to be used, but in turn

may induce cell aggregation and loss of electrolytes from the cell interior. A further limitation of free cell suspensions is cell sedimentation and convective overturning of the electrophoretic medium as a result of Joule's heating. Both these effects are related to gravity, and partial corrections can be made using density gradients; total correction necessitates a zero-gravity environment, such as pertains on board orbiting space vehicles. The latter approach shows great promise for high resolution, high yield electrophoretic separations of cells, and it is towards this ultimate goal that the present study is directed.

The greatest limitation to cell electrophoretic separations, however, is not susceptible to improved design of electrophoresis apparatus or even provision of a zero-gravity environment. Mixtures of functionally distinct cell populations, each possessing a unique configuration of surface groups, may nevertheless overlap to greater or lesser extents in their electrophoretic mobility distributions. Such populations are electrophoretically inseparable. Only by selectively modifying their surface charge density will cells of interest be susceptible to electrophoretic isolation.

The recent development of polymeric hydrophilic immunomicrospheres has allowed this approach to be tested (1). Conjugation of cell-surface specific antibodies to microspheres and subsequent coupling of the microspheres to cells

identifies or labels a specific cell population in a mixture. Furthermore, it has been shown (2) that the presence of microspheres on the cell surface significantly modifies the surface charge density of the cell. This selective modification of cell electrophoretic mobilities allows separation of otherwise inseparable cell mixtures, using conventional free-flow electrophoretic methods (2).

The present report summarizes studies conducted over the past year aimed at extending the previously reported red cell sepations using microspheres to purification of lymphocytes. Human peripheral blood lymphocytes are known to exist in two sub-groups, the thymus-derived T cells, and bursa-equivalent derived B cells (3). Cell surface structures have been used to identify the two sub-groups; T cells bear receptors for sheep red blood cells (4), B cells bear surface immunoglobulin (5) and complement receptor sites, and B and some T cells bear receptors for the F_c portions of immunoglobulins (6). These surface differences between T and B lymphocytes have prompted several workers to explore the separability of the sub-groups by electrophoresis (7-11) and isoelectric focusing (12, 13). While lymphocytes obtained from rodents were usually resolved into two peaks with considerable overlap, T cells showing slightly higher mobility, results with human lymphocytes were less encouraging (9, 14, 15). Electrophoretic mobility distributions were unimodal, with only partial enrichment of B and

T cells at the tail of the distribution. A microelectrophoretic study of human lymphocyte mobilities (16) concluded that T and B cells showed considerable overlap in their surface electrokinetic properties and that a significant degree of electrophoretic variability existed within individuals and also from person to person. A successful separation of B and T lymphocytes derived from human blood would have obvious research potential and clinical diagnostic advantages.

The availability of highly specific antisera to the surface immunoglobulins of human lymphocytes allowed us to label these cells with microspheres, and to show reductions in the electrophoretic mobility of lymphocyte suspensions so treated. Most significantly, selective labelling of the surface membrane immunoglobulin (Smig)-bearing lymphocytes (prospectively B cells) in preparations of peripheral blood lymphocytes generated bimodal mobility distributions, whereas untreated lymphocytes invariably showed unimodal distributions. Free-flow electrophoresis of labelled lymphocyte preparations resulted in partial separation of labelled from unlabelled lymphocytes; the optimal conditions of microsphere composition, cell labelling, and electrophoresis are under continuing investigation.

In the following sections of this report, four phases of the contract studies are presented. Firstly, the methodology and results of analytical mobility measurement of microspheres

are discussed. Secondly, analytical electrophoresis results with sheep red blood cells and their microsphere conjugates is presented, followed thirdly by the methodolgy and results of analytical and preparative electrophoresis of human lymphocytes. Finally, an account of recent experiments to produce monoclonal antibodies to hog gastric mucosal H^+-K^+ ATP-ase is presented. It is believed that used in conjuction with microspheres, these antibodies may prove useful in an improved purification of this enzyme by electrophoresis.

2. Microsphere Studies

2.1. Electrophoretic Mobility Measurement by Free-Flow Electrophoresis

Since the lower limit of visibility of particles in the cylindrical chamber microelectrophoresis apparatus (Figure 5) is 1 micron, free-flow electrophoresis was used for mobility measurement of microspheres in the size range 800-2000 Å. This method is characterized by a film of buffer flowing through a direct current electric field placed normal to the direction of flow. Charged particles injected into the buffer film as a narrow streak are deflected towards one or other of the electrodes, their migration distance depending on their surface charge density, the electric field strength, and buffer ionic strength and flow rate. The outflowing buffer film and deflected particles are directed through a multi-

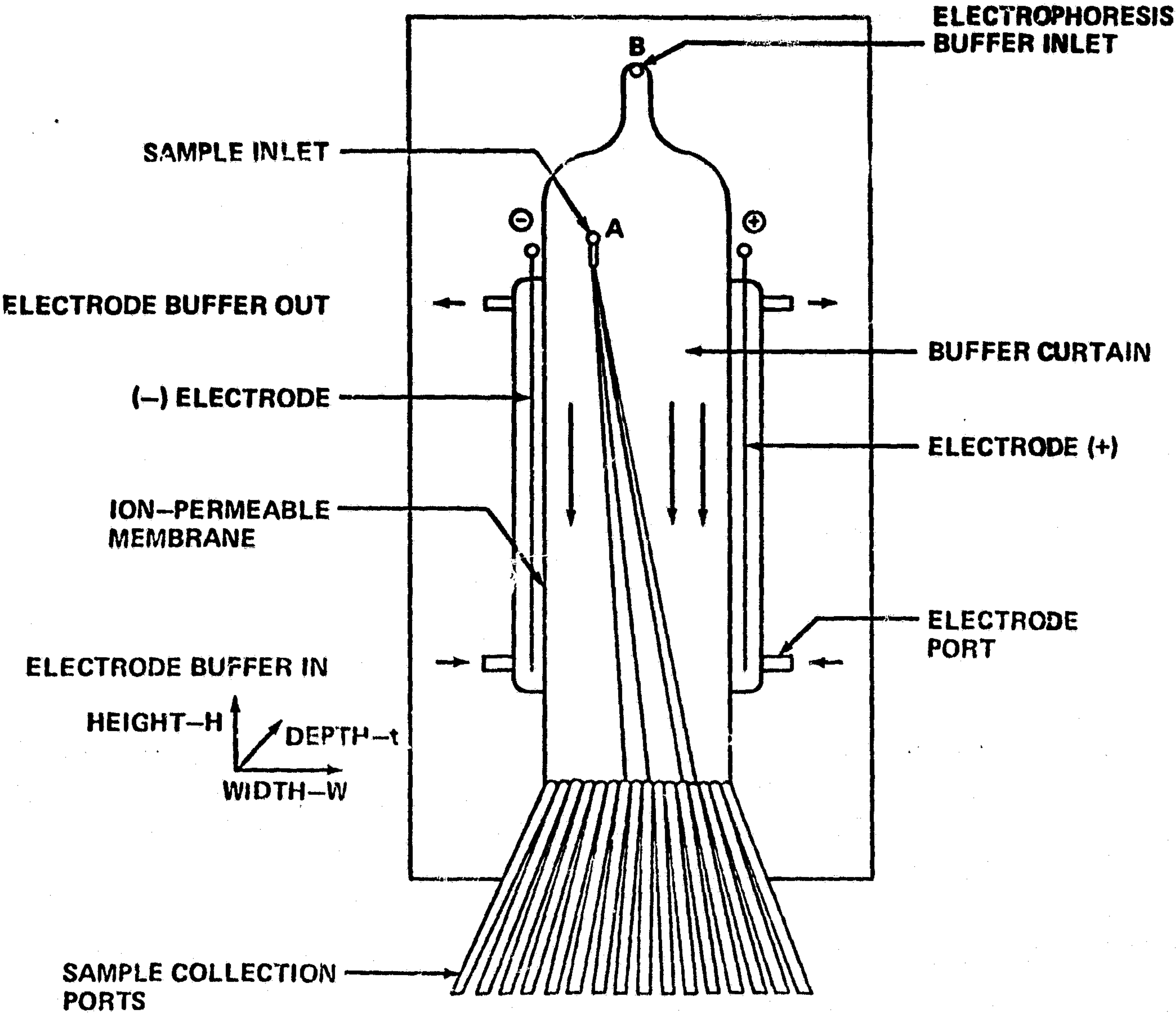

Figure 1. Schematic diagram of free-flow electrophoresis apparatus.

channel fractionator into collection tubes (Figure 1). Under standard conditions of field strength, buffer composition, and flow-rate, the lateral deflection of particles is proportional to their electrophoretic mobility, which can be estimated from calibration curves derived from free-flow electrophoresis of red blood cells under similar conditions. Figure 2 shows the variation of migration distance of red blood cells as a function of voltage gradient, and demonstrates the linearity of the electrophoretic response over a substantial voltage range. Figures 3 and 4 show the variation in migration distance of cells as a function of their electrophoretic mobility, determined independently by microelectrophoresis. The linearity of this plot and its extrapolation to zero mobility at zero field strength emphasizes the validity of mobility measurement by this means.

Microspheres were washed three times in electrophoresis buffer (Table 1) and resuspended in the same buffer at concentrations sufficient to give a well-defined sample streak in the free-flow electrophoresis chamber. Using a millmeter scale at the lower end of the chamber, microsphere migration distances under standard electrophoretic conditions were measured, and their mobilities estimated from Figures 3 and 4. The validity of this analytical method was further established by measurement of identical mobilities for 10 micron microspheres by cylindrical tube electrophoresis. The advantages

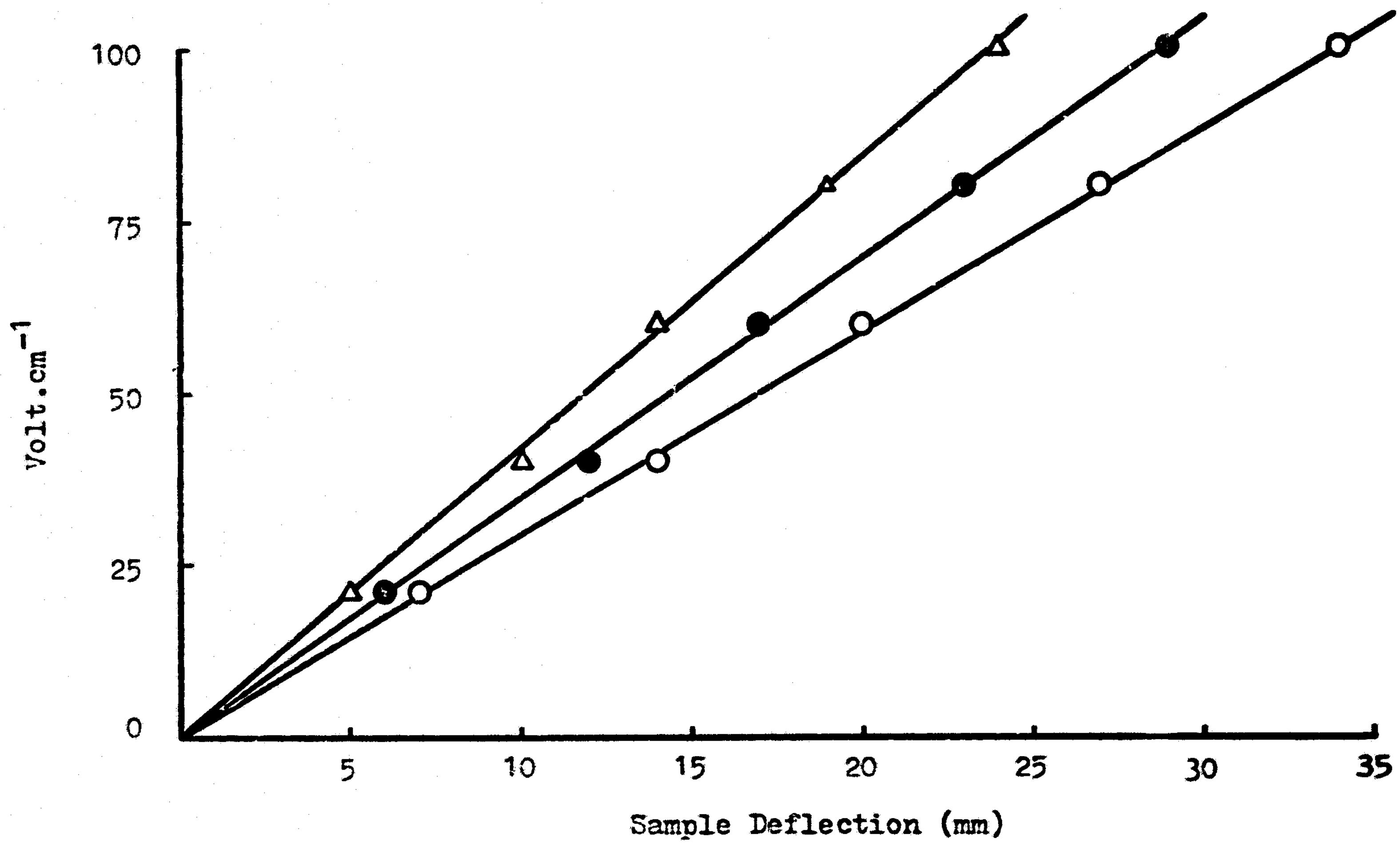

Figure 2. Electrophoretic Migration of Standard RBC as a function of Voltage gradient

Δ = Bovine RBC, ● = Human RBC, ○ = chicken RBC

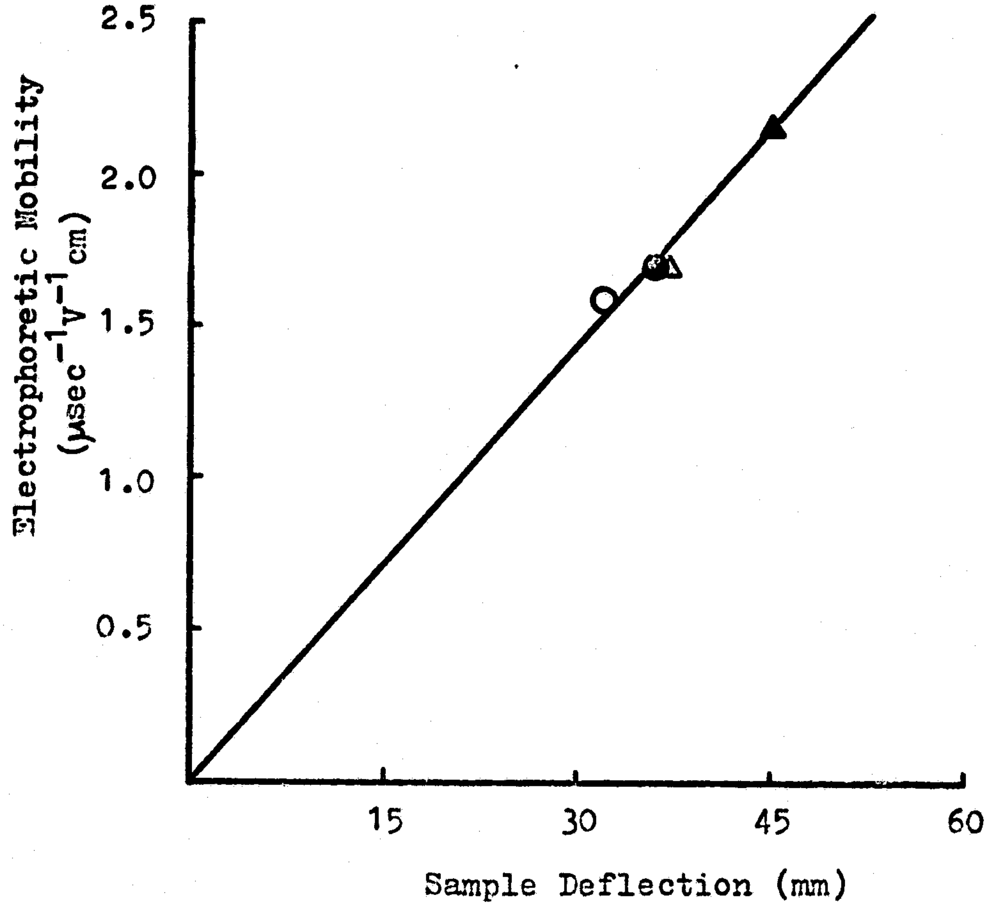

Figure 3. Calibration of FF-5 for mobility measurement using standard RBC in Tris-acetate electrophoresis buffer.

○ = Bovine RBC, ● = Human RBC △ = Sheep RBC
▲ = Chicken RBC

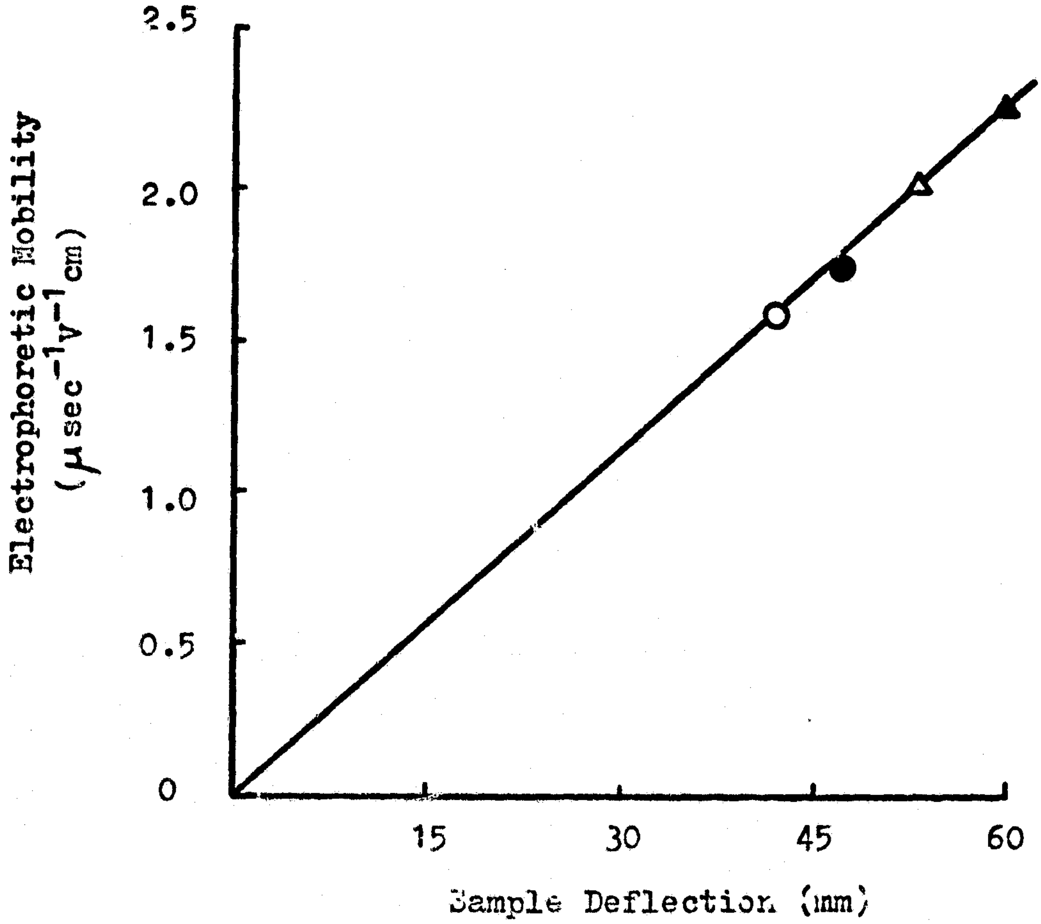

Figure 4. Calibration of FF-5 for mobility measurement using standard RBC in phosphate electrophoresis buffer.

▲ = Rat RBC, △ = Human RBC, ● = Bovine RBC

○ = Hybrid beads (cf. Table 2)

of the method are ease of electrophoretic screening of sub-microscopic particles, speed of mobility measurement, and reproducibility of results. The disadvantages are lack of precision owing to sample streak spreading, and the necessity of applying milligram quantities of microspheres per determination.

2.2. Operation of Free-Flow Electrophoresis Apparatus

The FF 5 apparatus (Desaga, Heidelberg, W. Germany) was used for preparative separation of labelled and unlabelled red blood cells and lymphocytes, and for analytical electrophoresis of sub-microscopic polygluteraldehyde and other microspheres. The operation of the apparatus is given in detail essential to reproducibility of results in both preparative and analytical studies.

The separation chamber was filled with 20% ethanol, and replaced after 10 minutes with 4 liters of distilled water passed through the chamber by gravitational siphoning from the electrophoresis buffer reservoir. The chamber was then drained and refilled with 2% bovine serum albumin. Albumin reduces the zeta potential at the walls of the separation chamber, which otherwise generates an electro-osmotic flow of buffer parallel to the wall towards the cathode. Electro-osmosis leads to sample streak cross-sectional distortion which results in remixing of separated fractions on collection (17). After 10 minutes,

the albumin solution was replaced by electrophoresis buffer flowing via the multi-channel peristaltic pump adjusted to the experiment flow rate. The electrode buffer pump was then switched on, taking care to eliminate air bubbles from the electrode compartments. The cooling unit was switched on, and when the chamber temperature reached 7.5°C, the electric field was established and raised in increments of 100 volts to the experiment voltage, ensuring that chamber temperature did not rise above 8.5°C. When voltage, current, and temperature were stabilized, sample injection was started at a maximal flow rate of 2.5 ml/hr. Following sample streak stabilization for 10 minutes, fraction collection was initiated and the run continued until the sample was exhausted. The cooling unit was switched off, and when the chamber temperature had reached 20°C, the voltage was reduced to zero by 00 volt decrements, keeping chamber temperature above 15°C. Electrophoresis buffer flow was discontinued, and the chamber flushed with 4 liters of distilled water. Electrode buffer was replaced with distilled water, ensuring that the return lines from the electrode compartments were fully immersed in the reservoir to prevent siphoning and subsequent drying of the ion-exchange membranes. The separation chamber was drained and refilled with 1% sodium dodecyl sulfate which was left overnight in the chamber, and then washed out with 4 liters of distilled water. Following this, the apparatus was ready

for another cycle of operation.

2.3. Free-Flow Electrophoresis Buffers

Two buffer compositions were used in the present study, and these are formulated in Table 1. The Tris-acetate buffer has previously been found to be optimal for the preparative free-flow electrophoretic separation of gastric membrane vesicles with preservation of their ionic transport functions (18). The phosphate buffer was formulated specially for separation of red blood cells by continuous flow electrophoresis (19). In both cases, the buffer ionic strength is substantially lower than physiological, in order to increase the electrophoretic mobility of sample cell populations by maximal extension of their electrophoretic double layers. Isotonicity of the buffers with biological cells was ensured by incorporation of 0.25 M sucrose. Both electrode buffers were 10 times more concentrated than the respective electrophoresis buffers, to minimize generation of Joule heat in the electrode chambers, and to minimize the voltage drop between the electrodes and the separation chamber.

2.4. Processing of Microspheres

The several classes of microspheres used in the present study were synthesized by Dr. Alan Rembaum at the Jet Propulsion Laboratory as described previously (20-22). Micro-

Table 1. Composition of Buffers for Microanalytical and Free-Flow Electrophoresis

Phosphate Electrophoresis Buffer*		Electrode Buffer*
$Na_2HPO_4 7H_2O$	1.89g	18.9g
KH_2PO_4	0.20g	2.0g
$Na_2EDTA.2H_2O$	0.57g	5.65g
Sucrose	342 g	-

*
pH = 7.4

Tris-Acetate Electrophoresis Buffer**		Electrode Buffer**
Trizma-Base	3.8g	48.44g
Glacial acetic acid	1.92g	24.02g
Sucrose	342g	-

** Adjust pH to 7.4 with 2N NaOH

Both buffers are made up to 4 liters with distilled water.

spheres, suspended in buffered aqueous media at concentrations of up to 20 mg/ml, were shipped to the Laboratory of Membrane Biology via overnight air freight service, where they were stored at 4°C until needed. Low ambient temperatures were maintained during shipping by wrapping the sample tubes in commercially available ice-packs (Divajex, Tustin, CA 92680). Microsphere samples were prepared for mobility measurement and cell separation as follows. The suspensions were vortexed vigorously for 30 seconds, sonicated for another 30 seconds (Model 112 SP 1T Ultrasonicator, Laboratory Supplies Co. Inc., Hicksville, N.Y.) and then centrifuged at 10,000rpm for 1 minute (Eppendorf Microfuge Model 3200). The supernatants were discarded, and the microsphere pellets resuspended in electrophoresis buffer by vortexing and sonication as above. Three washes in buffer were necessary to ensure electrokinetic stability of the microspheres; fewer washes resulted in disturbances of the microsphere sample streak on entering the free-flow electrophoresis chamber, accompanied by irreproducible measurements of electrophoretic mobility. Sonication was indispensable for formation of highly disperse microsphere suspensions in electrophoresis buffers of low ionic strength; microsphere dispersion was difficult or impossible to achieve when 0.15 sodium chloride or phosphate buffered saline was used as the suspension medium.

2.5. Electrophoretic Mobilities of Microspheres

Microsphere mobilities were measured as described in Section 2.1, at a field strength of 925 volts, 125 mA, and phosphate electrophoresis buffer flow rate of 9.3 ml/min. Microspheres with diameters in excess of 1 micron were also analyzed by microelectrophoresis in order to confirm the mobility value estimated from free-flow electrophoresis. The data shown in Table 2 illustrate the effects that differing ionogenic groups at the surface of a microsphere have on the electrophoretic mobility. Thus, large unsubstituted polystyrene microspheres showed very high negative mobilities; when susbstituted with amino groups which are extensively protonated at pH 7.4, the mobility of these microspheres dropped to $-1.15\,\mu sec^{-1}V^{-1}cm$, reflecting decreased net surface charge density. Small (2000 Å) polygluteraldehyde (PGL) microspheres showed a mobility of $-1.75\,\mu sec^{-1}V^{-1}cm$; when these microspheres were coupled to the larger amino-substituted beads, the electrophoretic mobility of the hybrid was $-1.58\,\mu sec^{-1}V^{-1}cm$. The result suggests that the mobility of the hybrid is dictated largely, but not exclusively, by the smaller microsphere. Table 3 shows the effect of lysine and glutamic acid on the mobility of PGL microspheres coated with goat anti-rabbit Ig antibody. The results suggest that both agents, when included in the polymerization medium, have insignificant effects on the mobility; however, when PGL microspheres were post-syn-

Table 2. Effect of Amino Substitution and Microsphere Hybridization on Mobility of Polystyrene Beads

Sample	Monomer	Diameter (microns)	Mobility ($\mu sec^{-1} V^{-1} cm$)
Polystyrene beads	Styrene	11	-5.69
Polystyrene-amine beads	Amino-substituted styrene	11	-1.15
DCM 207 microspheres	gluteraldehyde (3.5%v/v)	2	-1.75
Polystyrene-amine/207 hybrid beads		15	-1.58

Table 3. Effect of Lysine and Glutamic Acid on Mobility of PGL Microsphere-Goat anti-Rabbit Ig Conjugates

Sample Number	Addition to Polymerization Reaction Mixture: Glutamic acid	Addition to Polymerization Reaction Mixture: Lysine	Lysine Quench	Mobility ($\mu sec^{-1} V^{-1} cm$)
DCM 111	-	-	-	-1.54
DCM 160	+	-	-	-1.57
DCM 221	-	+	-	-1.65
DCM 162	-	-	+	-1.24

thetically quenched in lysine (1% w/v), a significant decrease in mobility resulted, reflecting the acquisition of positively charged groups which were not susceptible to neutralization by antibody. Incorporation of fluorescein isothiocyanate (FITC)-amine into PGL polymerization mixtures resulted in a similar reduction in electrophoretic mobility as a function of protonated amino groups at pH 7.4. The results of increasing FITC-amine concentration are shown in Table 4, and indicate ready saturation of the PGL microsphere matrix with FITC, at least as reflected in mobility measurements.

The effect of coupling goat anti-rabbit Ig antibody to hydroxy-ethyl-methacrylate/acrolein (HEMAC) microspheres on the mobility is shown in Table 5. The results show no consistent effect of antibody on microsphere mobilities, suggesting that the surface charge density of the microsphere conjugates was determined principally by HEMAC-FITC ionogenic groups. The anaomalous effect of FITC-amine in increasing the mobility of HEMAC microspheres is at present unaccounted for, but may be related to the greater susceptibility of HEMAC beads to swelling in aqueous media. Increases in microsphere size would in general increase their electrophoretic mobility, particularly in low salt media if double layer thickness was comparable to microsphere diameter (23).

Sample number DCM 253 (low salt) (Table 5) shows the lowest

Table 4. Effect of Fluorescein Isothiocyanate on Mobility of PGL Microsphere-Goat anti-Rabbit Ig Conjugates

Sample Number	Fluorescein Isothiocyanate in polymerization mixture (mg)	Mobility ($\mu sec^{-1} V^{-1}$ cm)
DCM 104	0	-1.92
DCM 113	5	-1.65
DCM 112	10	-1.54
DCM 111	15	-1.54

Table 5. **Mobility of Hydroxy-ethyl-methacrylate/Acrolein Microspheres**

Sample Number	Composition(%v/v)* HEMA	AC	MA	BA	Mobility ($sec^{-1}V^{-1}cm$) Native	+FITC	+FITC + antibody
DCM 245	40	60	-	-	-1.13	-1.27	-1.35
DCM 246	20	80	-	-	-1.13	-1.39	-1.39
DCM 247	30	70	-	-	-1.13	-1.35	-1.39
DCM 253	70	30	-	-	-0.93	-1.23	-1.38
DCM 253 (low salt)	"	"	-	-	-	-	-1.20
DCM 255	70	23	5	2	-1.04	-1.28	-1.38
DCM 255 (low salt)	"	"	-	-	-	-	-1.28
DCM 229**	30	-	10	30	-1.43	-1.58	-1.50

* HEMA = Hydroxy-ethyl-methacrylate
AC = Acrolein
MA = Methacrylic Acid
BA = Bis-acrylamide

** Also contains 30% acrylamide.

electrophoretic mobility of any microsphere-FITC-antibody conjugate screened to date, and is a promising candidate for future lymphocyte labelling and mobility modification.

3. Analytical Cell Electrophoresis

Red blood cell and lymphocyte electrophoretic mobilities were measured in a cylindrical tube apparatus fitted with silver/silver chloride electrodes, immersed in a constant temperature water bath at 25°C. A schematic diagram of the electrophoresis chamber is shown in Figure 5. The electrode compartments were filled with 0.145 M NaCl, buffered to pH 7.2 ± 0.2 with 0.5 M $NaHCO_3$ (standard saline), thereby eliminating problems due to KCl diffusion into the sample chamber and allowing samples to be reloaded several times without intermediate washing of the system. The electrical length of the electrophoresis chamber was measured in the usual way (24), using 0.01 M and 0.1 M KCl solutions of known conductivity. Before and after each set of red cell or lymphocyte mobility measurements, the accuracy of the apparatus was checked by electrophoresis of fresh human red blood cells suspended in standard saline. Only when consistent red cell mobilities of −1.08 ± 0.03 $\mu\,sec^{-1}V^{-1}cm$ were obtained (25) was the apparatus regarded as accurate and reliable. Electrophoretic observations were carried out in standard saline or in the electrophoresis buffers shown in Table 1. Cells to be meas-

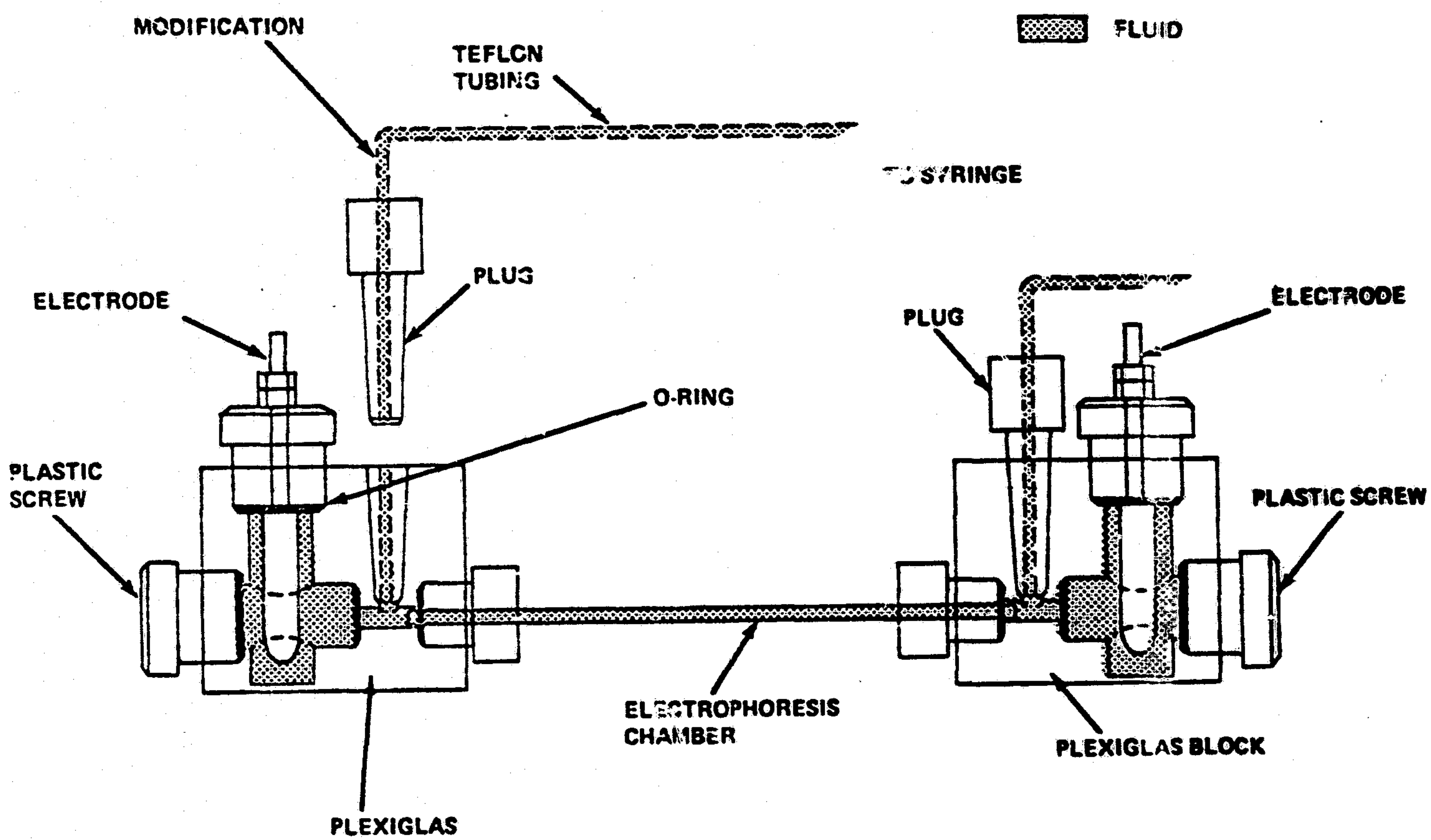

Figure 5. Schematic diagram of analytical micro-electrophoresis chamber with electrodes.

ured were washed twice in the appropriate buffer and resuspended in the same buffer at a concentration of 2×10^6 cells/ml. The applied field strength was 4.0 volts/cm. Only those cells in focus at the stationary level with typically small lymphoid morphology or typical biconcave disc morphology in the case of red cells were timed; larger cells similar to monocytes or neutrophils were not timed. At least 50 individual cells were timed in both directions for each sample distribution, in order to eliminate convective or gravitational drifts and to minimize electrode polarization effects. Times of migration were recorded using a Micronta electronic stop-watch accurate to 0.01 seconds, and these times were entered into a preprogrammed Compucorp 325 Calculator for print-out of electrophoretic mobilities, coefficients of variance, and standard deviations from the mean. For each sample distribution the electrophoretic mobilities were collated in frequency histogram form, showing the percentage of cells in standard mobility intervals.

4. Sheep Red Blood Cell Studies

4.1. Background

Modification of cell electrophoretic mobility by immunospecific coupling of microspheres to the cell plasma membrane is most conveniently studied with red blood cells, which are readily available in large quantities, are easily purified, can be

rendered osmotically insensitive by treatment of aldehydes without loss of characteristic electrokinetic properties, and to which highly specific cell surface binding antisera are available. Sheep red blood cells were selected at the Jet Propulsion Laboratory as a model cell population to confirm the previously observed separability of human red blood cells by means of microspheres (2), while analytical mobility studies of derivatized SRBC were performed at the Laboratory of Membrane Biology.

Three classes of microspheres were investigated for their effects on the electrophoretic mobility of SRBC. PGL microspheres were synthesized in the presence or absence of lysine in the polymerization mixture; the occurence of protonated e-amino groups at pH 7.4 could be expected to confer some degree of positive surface charge to microspheres incorporating lysine, thereby lowering their net negative surface charge density. Microspheres were also incubated after synthesis in 1% lysine in an attempt to confer analogous reductions in electrokinetic net charge by adsorption of amino groups to the surface and interstices of the PGL matrix. A third group of microspheres was incubated after synthesis in 1% glutamic acid; the presence of free e-carboxyl groups could be expected to increase the the net negative surface charge of these microspheres. Corresponding modifications of electrophoretic mobility of SRBC labelled with the three classes of

microspheres were sought by microelectrophoresis.

4.1.1. SRBC Sensitization and Labelling

SRBC were stored in sterile Alsevers medium at 4°C until used. They were washed three times in phosphate buffered saline (PBS) at pH 7.4 and resuspended at 10^9 SRBC/ml. To 1 ml of this suspension was added 1 ml of a 1:20 dilution in PBS of rabbit anti-SRBC antibody, followed by gentle agitation at 37°C for 30 minutes. The cells were washed three times in PBS to remove unbound antibody, then resuspended and washed twice in phosphate electrophoresis buffer to a final concentration of 2×10^8 cells/ml. To 2 ml of the suspension were added 2 ml of a 2 mg/ml suspension of either lysine-incorporated, lysine-quenched, or glutamic acid quenched PGL microspheres conjugated to goat anti-rabbit Ig antibody. The cell suspensions were incubated with gentle agitation at 25°C for 1 hour, then washed twice in electrophoresis buffer to remove unbound microspheres. Control SRBC were prepared similarly with the exception that PBS replaced rabbit anti-SRBC antibody in the sensitization step.

4.2. Mobilities of Labelled and Unlabelled SRBC

Representative electrophoretic mobility distributions of normal, sensitized, and microsphere labelled SRBC are shown in Figure 6. The mobilities of normal and sensitized SRBC

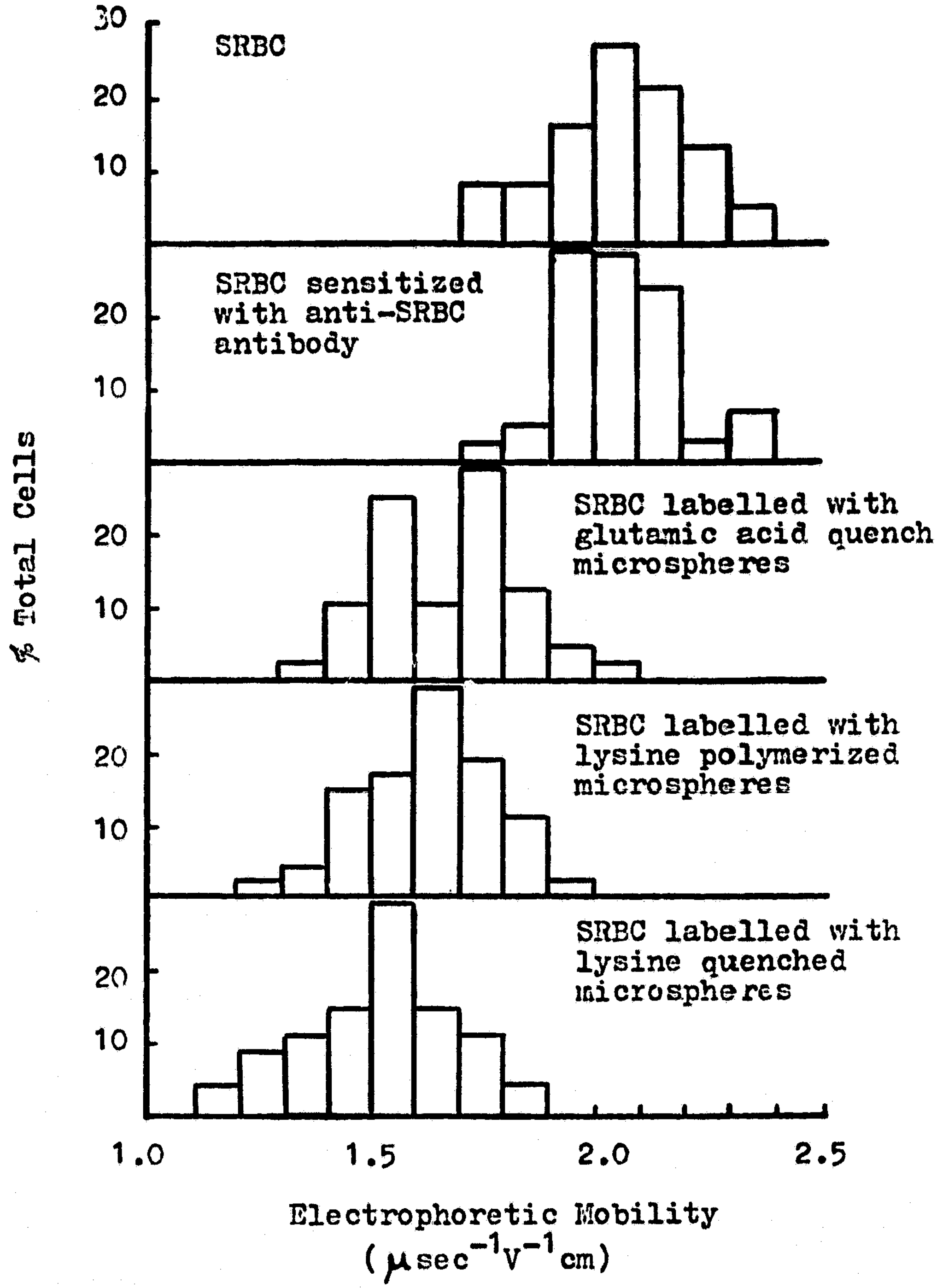

Figure 6. Electrophoretic mobility distributions of normal, sensitized, and PGL microsphere labelled SRBC in Tris-acetate buffer.

were not significantly different, being -2.06 ± 0.15 and $-2.05 \pm 0.12\,\mu sec^{-1}V^{-1}cm$ respectively. Labelling of sensitized SRBC with glutamic acid quenched microspheres reduced their mean mobility to $-1.67 \pm 0.16\,\mu sec^{-1}V^{-1}cm$, with emergence of a bimodal mobility distribution in contrast to the unimodal distribution exhibited by normal or sensitized SRBC. This electrophoretic splitting of the cells was unaccounted for, but could be due to incomplete or differential derivatization of the microspheres with glutamic acid, giving rise to an electrophoretically heterogeneous microsphere population.

SRBC labelled with lysine-incorporated or lysine quenched microspheres showed unimodal mobility distributions, with mean mobilities of -1.62 ± 0.15 and $-1.51 \pm 0.18\,\mu sec^{-1}V^{-1}cm$ respectively. These results confirmed that the greatest reduction in cell mobilities resulted from derivatization of microspheres with e-amino groups. The fact that glutamic acid-quenched microspheres reduced SRBC mobility despite the presence of negatively charged groups donated by the e-carboxyls of glutamic acid suggested either that insufficient of these had been bound, or that microsphere ionogenic groups were not solely responsible in determining the net surface charge density of labelled cells. The possibility that goat anti-rabbit Ig antibody ionogenic groups contribute in some degree to the surface charge density cannot be excluded, although

earlier results of microsphere mobility studies (Table 5) indicated minimal differences in the mobilities of fresh and immunoglobulin-conjugated microspheres of widely divergent compositions.

4.3. Mobility of SRBC-microsphere Conjugates as Function of pH

Separation of cells by isoelectric focusing has been described by several authors (12, 13, 26). The method is based on the electrophoretic migration of cells through a pH gradient, established either by prefocusing of Ampholines (LKB, Bromma, Sweden) or by means of a discontinuous gradient of appropriate buffer ions. Since the electrophoretic mobility of cells is a function of their surface charge density, or degree of dissociation of membrane ionogenic groups, their mobility is closely related to the pH of the electrophoretic medium. At a certain usually low pH the net surface charge of a cell equals zero, at which point it ceases to migrate electrophoretically. Cell populations exhibiting heterogeneity in isoelectric point are therefore in principle separable by isoelectric focusing, since sub-populations with different isoelectric points will concentrate in different regions of the pH gradient. In practice the separation is difficult to achieve because of aggregation and loss of viability of cells when exposed to their isoelectric pH. The availability of microspheres, their

ready and highly specific attachment to selected cell subpopulations, and their significant modification of the cell's electrophoretic response, prompted us to investigate their utility in cell separation by isoelectric focusing. It was thought that cell labelling with microspheres may shift the cell isoelectric point to a range which is less inimical to cell viability, thereby extending the usefulness of cell focusing methods.

In preliminary experiments, sheep red blood cells were labelled with PGL microspheres and their mobility subsequently measured as a function of pH. SRBC were washed thoroughly in PBS at pH 7.4 and resuspended in the same at a concentration of 4×10^8 SRBC/ml. 0.5 ml of 1:100 dilution of rabbit anti-SRBC antibody in PBS was added to 1 ml of cells, and incubated for 30 minutes at 37°C. The cells were washed twice in PBS then twice in phosphate electrophoresis buffer to a final volume of 4 ml. 1 mg of lysine-quenched PGL microspheres (DCM 158), derivatized with goat anti-rabbit Ig antibody, and suspended in 1 ml phosphate electrophoresis buffer, was then added to the cells, and incubated for 1 hour at 25°C. After two final washes in electrophoresis buffer at pH 7.4, the cells were resuspended to 8 ml and their mobility measured. Five additional SRBC samples were treated similarly, except that the final washes were carried out in phosphate electrophoresis buffers with pH adjusted to 6.3, 5.3, 4.3, 3.3, and 2.4 res-

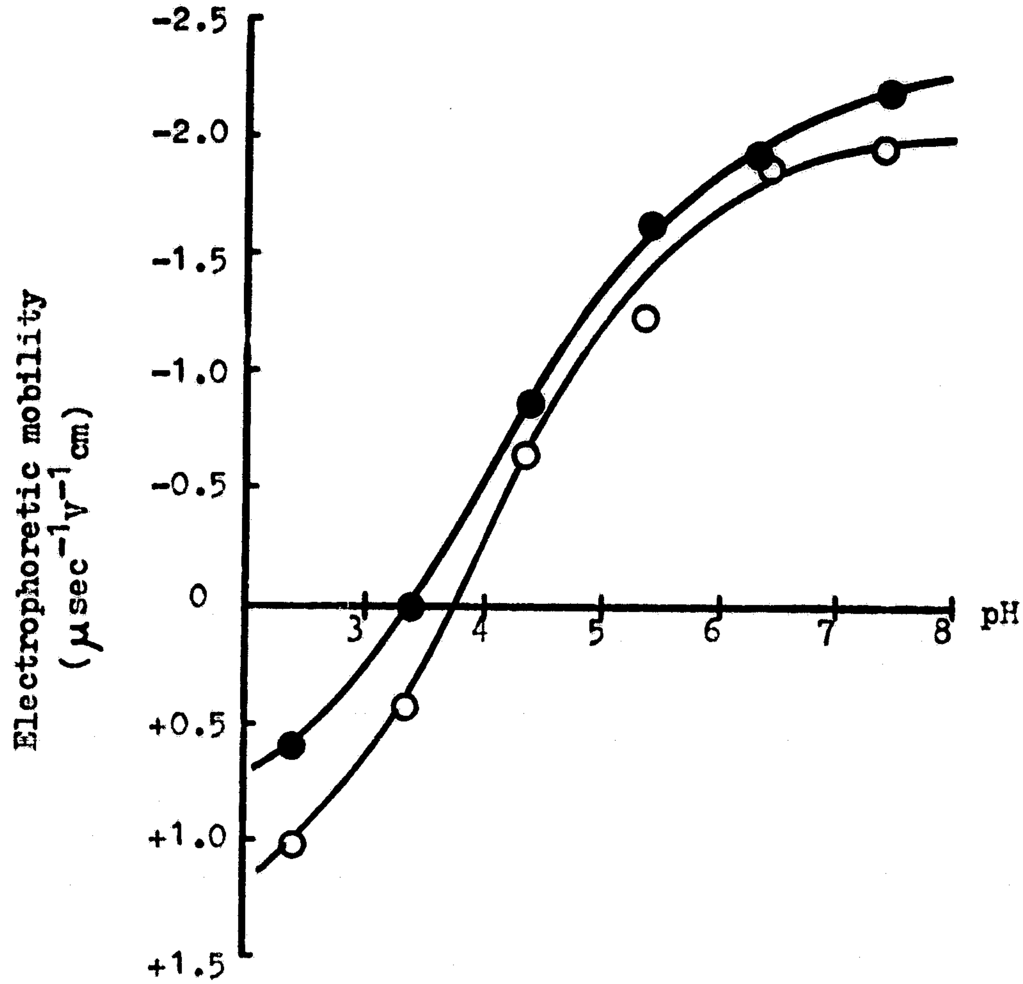

Figure 7. Electrophoretic mobility of normal SRBC (●) and PGL microsphere labelled SRBC (○) as a function of pH, phosphate electrophoresis buffer.

pectively. Binding of the fluorescent microspheres to the SRBC was confirmed by fluorescence microscopy. In the preparation of control SRBC, the above protocol was followed, except that in the cell sensitization step, the rabbit anti-SRBC antibody was replaced with PBS. These cells showed no binding of fluorescent microspheres. Control cells' electrophoretic mobilities were identical to those measured for fresh SRBC suspended in electrophoresis buffers at all the pHs tested.

The mobility dependency of SRBC and microsphere labelled SRBC on pH is shown in Figure 7. The results indicated that the isoelectric pH of normal cells was approximately 3.3, while that of microsphere labelled cells was 3.8. Both labelled and normal cells showed extensive aggregation at pHs lower than 4.0. Nevertheless, the small shift in cell isoelectric point suggests that microspheres could be effective in cell modification for isoelectric focusing separations, although further studies are needed to clarify this point.

5. Lymphocyte Studies

5.1. Background

Human lymphocytes were labelled with microspheres by the indirect antibody technique, whereby the cells were first exposed to rabbit antisera specific for lymphocyte plasma membrane receptors, and then exposed to microspheres conjug-

ated with goat anti-rabbit Ig antibody. Two groups of rabbit antisera to human lymphocytes were used. The first group, represented by rabbit anti-human lymphocyte antiserum (ALS), showed reactivity against all human lymphocytes regardless of cell type, being unable to distinguish between the T and B cell sub-groups present in normal peripheral blood lymphocytes. The second class of antisera consisted of several ammonium sulfate precipitated and affinity purified rabbit antibodies showing specificity for either all human lymphocyte surface membrane immunoglobulins (Smig) or specific classes of Smig such as IgM, IgG, IgD, IgE, and the kappa or lambda light chain variants of these classes. Thus the second group of antisera recognized lymphocytes belonging to the B cell sub-group. T lymphocytes are not labelled by these antisera, an observation which forms the basis for differentiating lymphocytes into T and B cell sub-groups (4, 5).

Rabbit ALS was used in the present study to assess the feasibility of modifying the electrophoretic mobility of lymphocytes by microsphere labelling, and to exploit the mobility difference by separating labelled from unlabelled lymphocytes. The anti-Smig antisera were used to modify the electrophoretic mobility of the B lymphocytes alone, and to assess subsequently the separability of the B cells from peripheral lymphocytes by free-flow electrophoresis.

5.2. Preparation of Lymphocytes

The separation of lymphocytes from whole blood was based with slight modifications on the procedure of Boyum (27). Human peripheral venous blood was collected from healthy donors into sterile syringes containing 10 I.U. of heparin per ml of blood. After gentle agitation to ensure mixing, the blood was diluted with two volumes of Ca^{2+} and Mg^{2+} free PBS in a siliconized Ehrlenmeyer flask. 7.5 ml of Ficoll-Paque solution (Pharmacia, Uppsala, Sweden) was pipetted into several 35 ml siliconized plastic centrifuge tubes, as required. 11 ml of PBS-diluted blood was layered carefully onto the Ficoll-Paque solution , and the tubes were centrifuged at 400 g for 40 minutes at 25°C (Sorvall GLC-2B, 1500 rpm). The interfacial cells were collected by Pasteur pipette into 15 ml siliconized polystyrene centrifuge tubes, and diluted with one-quarter their volume of Ca^{2+} and Mg^{2+} free Hank's balanced salt solution, followed by centrifugation at 100 g for 10 minutes (Sorvall, 1000 rpm). The supernatants, containing platelets, plasma, and Ficoll-Paque, were discarded, and the lymphocyte pellets resuspended in 5 ml RPMI 1640 tissue culture medium (Grand Island Biological Co., N.Y.) followed by centifugation at 100 g for 10 minutes. After one more wash in RPMI 1640, the lymphocytes were resuspended in 1 ml of the same medium and counted using an AO Bright Line hemocytometer (Scientific Instruments Division, Buffalo, N.Y.)

Viability of the lymphocytes was estimated by trypan blue exclusion, whereby a drop of the lymphocyte suspension was mixed with one drop of 0.4% trypan blue in saline, incubated for 5 minutes under the hemocytometer cover slip, and examined by phase contrast microscopy at 400 x magnification. Cells staining blue were considered to be non-viable.

In routine use, the above procedure yielded suspensions of mononuclear cells with characteristic small lymphoid morphology, with yields from whole blood of approximately 50% and viability in excess of 95%. When suspended in RPMI 1640 tissue culture medium at $4^{o}C$ at concentrations of 10^6 cells/ml, the viability of the lymphocytes was 90% after 24 hours, although after 36 hours the viability had decreased to 50%. Consequently, further lymphocyte manipulations such as analytical mobility measurement, sensitization with antibodies, microsphere coupling, or free-flow electrophoresis, were carried out immediately after their separation from blood, generally at $4^{o}C$ whenever practical.

5.2.1. Lymphocyte Sensitization

Lymphocytes were centrifuged at 1000 rpm for 10 minutes, and the supernatants discarded. 10 ul of undiluted rabbit ALS or rabbit anti-Smig antibody was added to the moist lymphocyte pellet for every 10^6 cells present. All antisera were centrifuged at 120,000 g for 20 minutes in an ultracentrifuge (Airfuge,

Beckman Instruments Inc., CA 94304) immediately before use to remove aggregated IgG. This precaution was found to give more reproducible labelling and to minimize agglutination of lymphocytes. The cells were kept on ice with occasional tapping of the cell pellet to ensure mixing of the tube contents. After 30 minutes, the cells were diluted with 5 ml of RPMI 1640 and centrifuged at 100 g for 10 minutes. Supernatant was discarded, and the cells washed twice more in RPMI 1640 to ensure removal of unbound antibody. Examination of the cells by fluorescent microscopy was carried out to estimate the relative numbers of labelled and unlabelled lymphocytes. In those cases where lymphocytes were sensitized with non-fluorescent antibodies, control cells were labelled with the identical antibody coupled to fluorescein isothiocyanate in order to confirm and quantitate lymphocyte labelling.

Lymphocytes were then transferred to Tris-acetate or phosphate electrophoresis buffers by gradual two-stage replacement of the RPMI 1640 by centrifugal washing at 100 g for 10 minutes. This step was essential prior to labelling of cells with microspheres, otherwise the high salt concentration of RPMI 1640 would cause immediate and extensive aggregation of microspheres and lymphocyte-microsphere conjugates.

Table 6 shows the percentage of human lymphocytes labelled with fluorescent rabbit ALS and anti-Smig antisera using the

Table 6. <u>Surface Staining of Human Lymphocytes with Fluorescent Rabbit Anti-Sera.</u>

FITC-Antisera	% Lymphocytes stained
1. Anti-lymphocyte serum (ALS)	75-80
2. Anti-human Ig (light chain, lambda)	8
3. Anti-human Ig (light chain, kappa)	10
4. Anti-human IgM (μ chain)	9-10
5. 1:1 mixture of antisera 2 and 3 above (rabbit anti-Smig)*	16-20

* Smig = Surface membrane immunoglobulin.

above protocol.

5.2.2. Lymphocyte Labelling with Microspheres

Microspheres used for cell surface labelling were conjugated with goat anti-rabbit Ig antibody at the Jet Propulsion Laboratory as described previously (20-22). PGL microspheres were derivatized by simple incubation with antibody followed by blocking of unreacted sites with glycine, while HEMAC beads were derivatized by carbodiimide bridging with the antibody. The microspheres were then washed twice and resuspended in electrophoresis buffer at appropriate concentrations determined as follows.

The surface area of a lymphocyte is approximately 200 um^2, and the cross-sectional area of a single microsphere is about 0.03 um^2. Therefore, assuming complete coverage of the cell surface, 6,600 microspheres will label one cell, or 6.6×10^9 microspheres per million cells. One mg of microspheres comprises approximately 10^{12} microspheres, so that 10 ug microspheres should be sufficient to label 10^6 cells. In practice, 100 ug microspheres were used per 10^6 lymphocytes in order to ensure maximal binding to the cells.

Equal volumes of lymphocytes and microspheres, both suspended in electrophoresis buffer, the former at concentrations not exceeding 10^7 cells/ml, were combined and incubated with frequent gentle vortexing for 1 hour at 4^oC. The cell-microsphere suspension was then washed twice in electrophoresis

buffer at 80 g for 7 minutes; more extensive centrifugation tended to precipitate unbound microspheres and promote aggregation of labelled lymphocytes. After the final wash, the lymphocytes were resuspended at 10^7 cells/ml in electrophoresis buffer, small aliquots were taken for examination by fluorescence microscopy to determine extent of labelling, and the remainder used immediately for mobility measurements or free-flow electrophoresis.

The relative proportions of microsphere labelled and unlabelled lymphocytes as estimated by fluorescence microscopy correlated closely with the relative proportions of surface stained lymphocytes using the same FITC-rabbit antisera as used for microsphere coupling. However, some difficulty was experienced in obtaining dispersed lymphocyte suspensions following microsphere labelling in the low ionic strength buffers essential to electrophoresis. Labelled cells showed considerable aggregation and pronounced loss of viability especially after free-flow electrophoresis. The aggregation may be caused in part by activation of lymphocytes triggered by indirect antibody labelling, with release of endogenous antibodies followed by cell rupture. An alternative approach currently under investigation is direct antibody labelling of lymphocytes, whereby microspheres coupled to anti-Smig antibody interact directly with lymphocyte membrane receptors. It is hoped that this technique may

alleviate cell agglutination and provide the added benefit of simplifying lymphocyte labelling.

5.3. Lymphocyte Electrophoresis

5.3.1. Electrophoretic Mobilities

The electrophoretic mobility of lymphocytes was measured as described in Section 3. Figure 8 is a composite of four lymphocyte mobility distributions showing that sensitization of lymphocytes with either rabbit ALS or rabbit anti-Smig antibody had no significant effect on cell electrophoretic mobility. Similarly, non-sensitized lymphocytes exposed to PGL microspheres conjugated with goat anti-rabbit Ig antibody showed no change in mobility, indicating absence of non - specific binding of microspheres to the cells.

In contrast, Figure 9 shows the effect of microsphere binding to the lymphocyte surface on the cell mobility distribution. While the control mean electrophoretic mobility was $-1.17 \pm 0.07\,\mu sec^{-1}V^{-1}cm$ (a), the value for lymphocytes sensitized with rabbit ALS and then labelled with microspheres was $-0.89 \pm 0.16\,\mu sec^{-1}V^{-1}cm$. The mean mobilities of cells sensitized with rabbit anti-Smig antibody and labelled with microspheres was $-1.00 \pm 0.23\,\mu sec^{-1}V^{-1}cm$. Of special interest is the unimodal mobility distribution observed for untreated lymphocytes in Figures 8 and 9, compared to the bi-modal distributions evident in microsphere-labelled lympho-

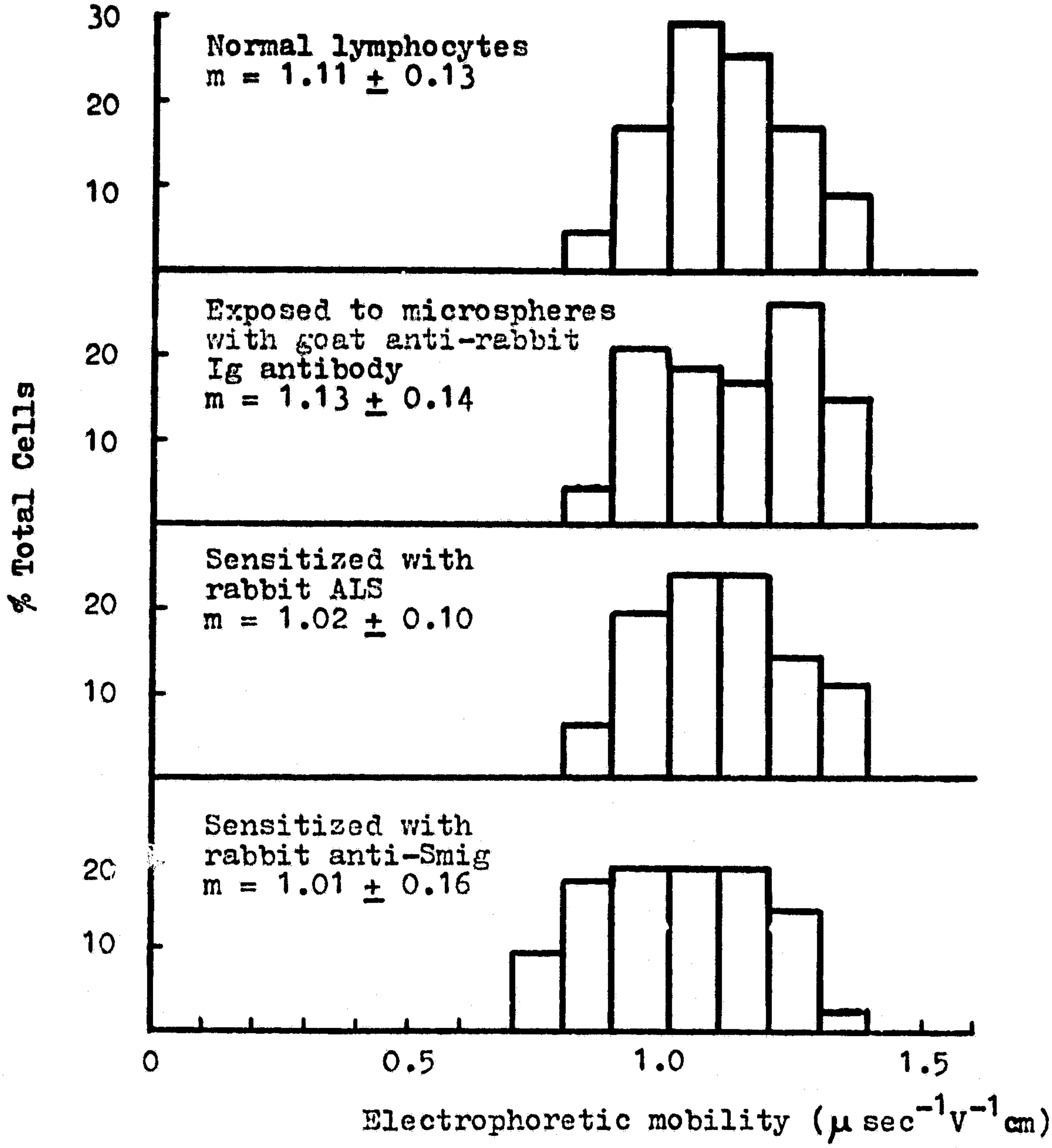

Figure 8. Electrophoretic mobility distributions of normal and sensitized human lymphocytes in standard saline.

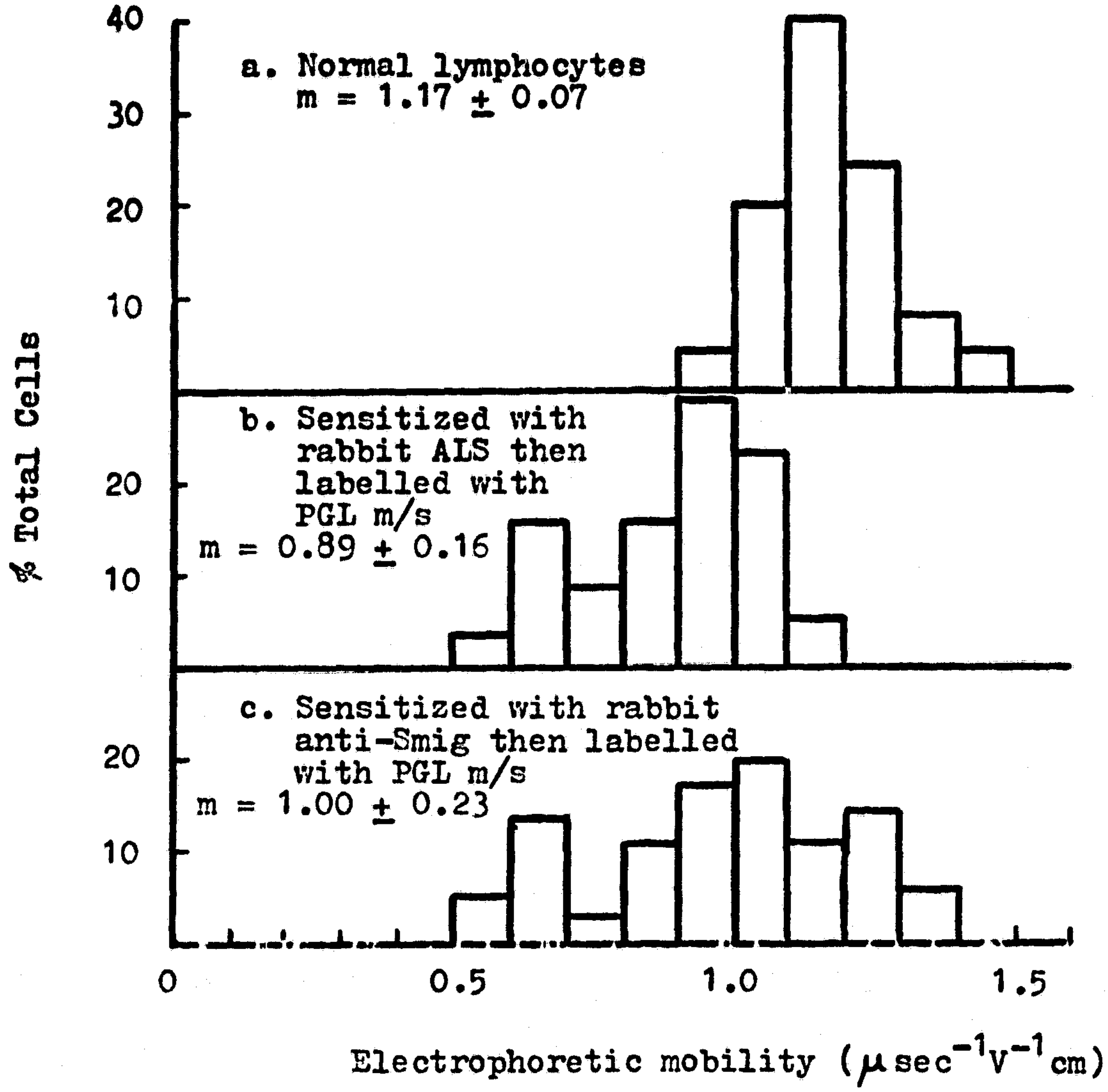

Figure 9. Electrophoretic mobility distribution of human lymphocytes labelled with PGL microspheres, in standard saline.

cytes. While the entire distribution of ALS sensitized and microsphere labelled cells is shifted to lower mobility (Figure 9, b and c), only a small fraction of the anti-Smig sensitized cells show an equivalent shift, since presumably only B cells were affected by the labelling procedure in the latter case.

Figure 10 shows the mobility distributions for untreated SRBC and lymphocytes suspended in the Tris-acetate buffer. Both distributions were unimodal, and their mean mobilities were -2.06 ± 0.15 and $1.71 \pm 0.19 \mu sec^{-1}V^{-1}cm$ respectively. When both cell types were sensitized with rabbit antibodies then labelled with goat anti-rabbit Ig conjugated PGL microspheres, both mean mobilities were lowered, coinciding at $\sim -1.5 \mu sec^{-1}V^{-1}cm$. This result suggested that the modifications of cell mobility induced by microspheres were generated by the surface charge density of the microsphere-antibody complex, with minimal contributions from the membrane associated ionogenic groups of the cells themselves. This interpretation is supported by the mobilities of PGL microsphere-antibody conjugates, measured by free-flow electrophoresis, which range from -1.3 to $-1.7 \mu sec^{-1}V^{-1}cm$.

5.3.2. Preparative Lymphocyte Electrophoresis

When untreated human lymphocytes were processed by free-flow electrophoresis, there was no visible splitting of the sample

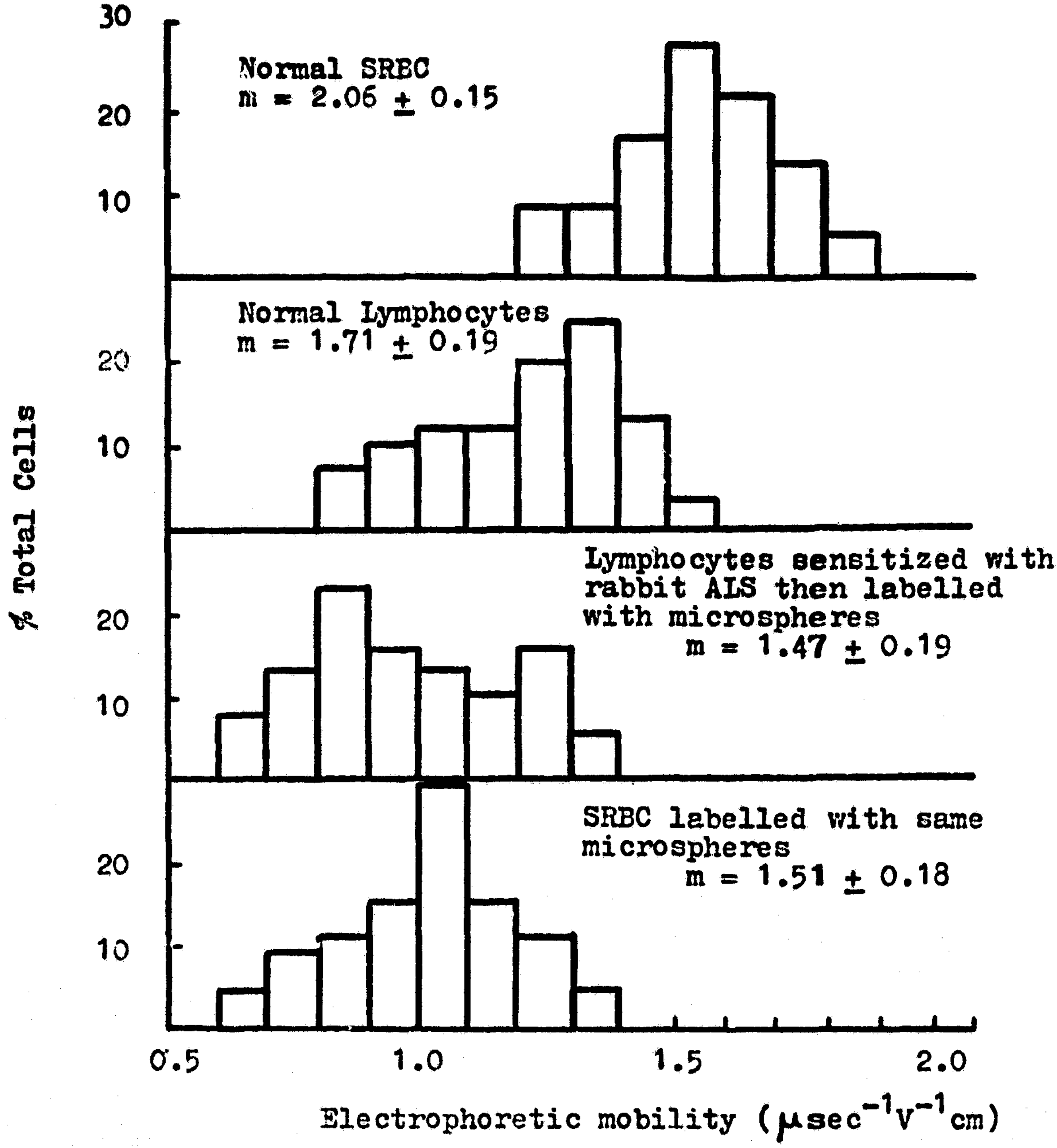

Figure 10. Electrophoretic mobility distributions of SRBC and human lymphocytes labelled with PGL microspheres, in Tris-acetate electrophoresis buffer.

streak in the electrophoresis chamber, and the cells were collected in an essentially unimodal distribution spread over ~10 fractions depending on the buffer flow rate and electrical field strength. Fresh lymphocytes were sensitized with rabbit ALS and then labelled with fluorescent PGL microspheres (DCM 158) with mobility = $-1.58 \mu sec^{-1} V^{-1} cm$. Equal numbers of labelled and unlabelled lymphocytes were combined in Tris-acetate buffer, washed once, and then processed by free-flow electrophoresis at a buffer flow rate of 6 ml/min. Marked splitting of the lymphocyte sample streak was apparent as the cells traversed the length of the separation chamber; total and differential (fluorescent/non-fluorescent) lymphocyte counts of the collected fractions revealed the cells to be spread over 16 tubes, with the distribution shown in Figure 11. Fluorescent lymphocytes, ie., those labelled with microspheres, were found closer to the cathode than untreated (non-fluorescent) lymphocytes, indicating slower electrophoretic mobilities among the labelled cells, in accordance with the findings of microelectrophoretic analysis of lymphocyte mobilities discussed earlier. Approximately 40% of the recovered labelled lymphocytes were contaminated with unlabelled lymphocytes, and showed fairly extensive aggregation , unlike the untreated cells which were recovered in monodisperse form. The remaining labelled lymphocytes were free of untreated cells, but also showed extensive

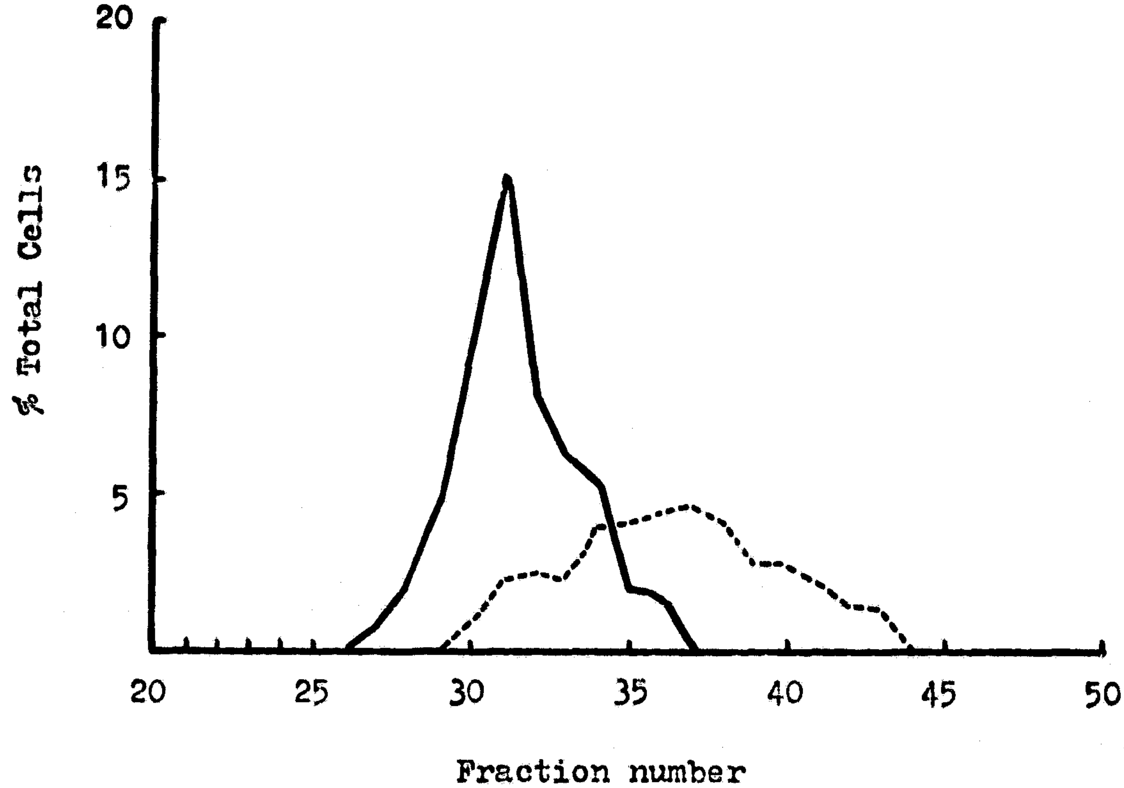

Figure 11. Free-flow electrophoretic separation of normal lymphocytes (——) from rabbit ALS sensitized lymphocytes labelled with fluorescent PGL microspheres (-----). Tris-acetate electrophoresis buffer.

clumping and 80% loss of viability.

In another experiment, lymphocytes were sensitized with rabbit anti-Smig antibody, and subsequently labelled with fluorescent PGL microspheres (DCM 111) showing an electrophoretic mobility of $-1.54 \mu sec^{-1} V^{-1} cm$. The lymphocytes were processed as before (120 volts/cm, 6 ml/min buffer flow rate) and the results of total and differential cell counts of recovered fractions are shown in Figure 12. Labelled cells constituted $\sim$13% of the total lymphocyte count, and about 50% of these were recovered free of untreated lymphocytes. Once again, labelled cells showed a high degree of aggregation and loss of viability.

The experiment was repeated, using DCM 158 fluorescent PGL microspheres, at a buffer flow rate of 10 ml/min. The faster flow rate diminished the residence time of cells in the electric field, and thus exposure of cells to electrophoresis buffer; in addition, processed cells were collected in 2 ml RPMI 1640 tissue culture medium, and washed once in this medium before analysis. The distribution shown in Figure 13 was obtained, which indicates labelled lymphocytes occupying the cathodic side of the cell distribution with considerable enrichment of those cells in fractions 46, 47, and 48. Labelled cells showed less aggregation than in previous experiments, with lymphocyte viabilities in excess of 60%.

Figure 14 shows the lymphocyte distribution in two further

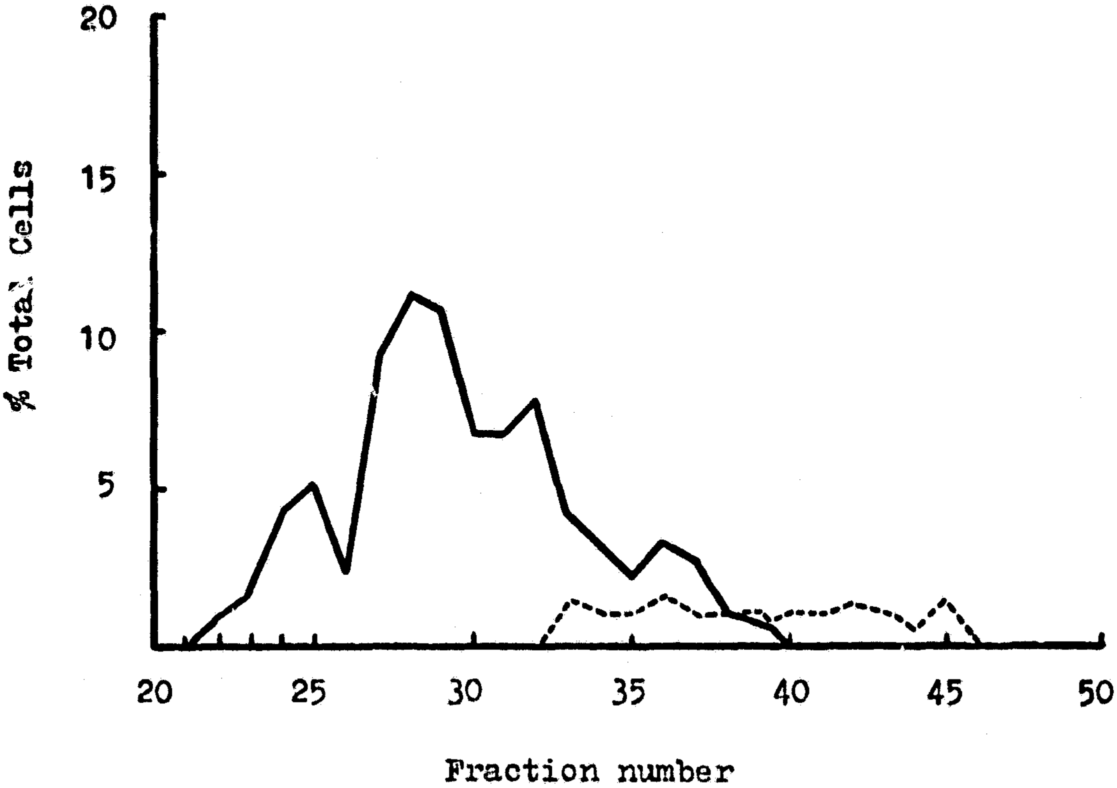

Figure 12. Free-flow electrophoretic separation of lymphocytes sensitized with rabbit anti-Smig then labelled with fluorescent PGL microspheres (-----) from unlabelled lymphocytes (——). Acetate electrophoresis buffer flow rate = 6 ml/min.

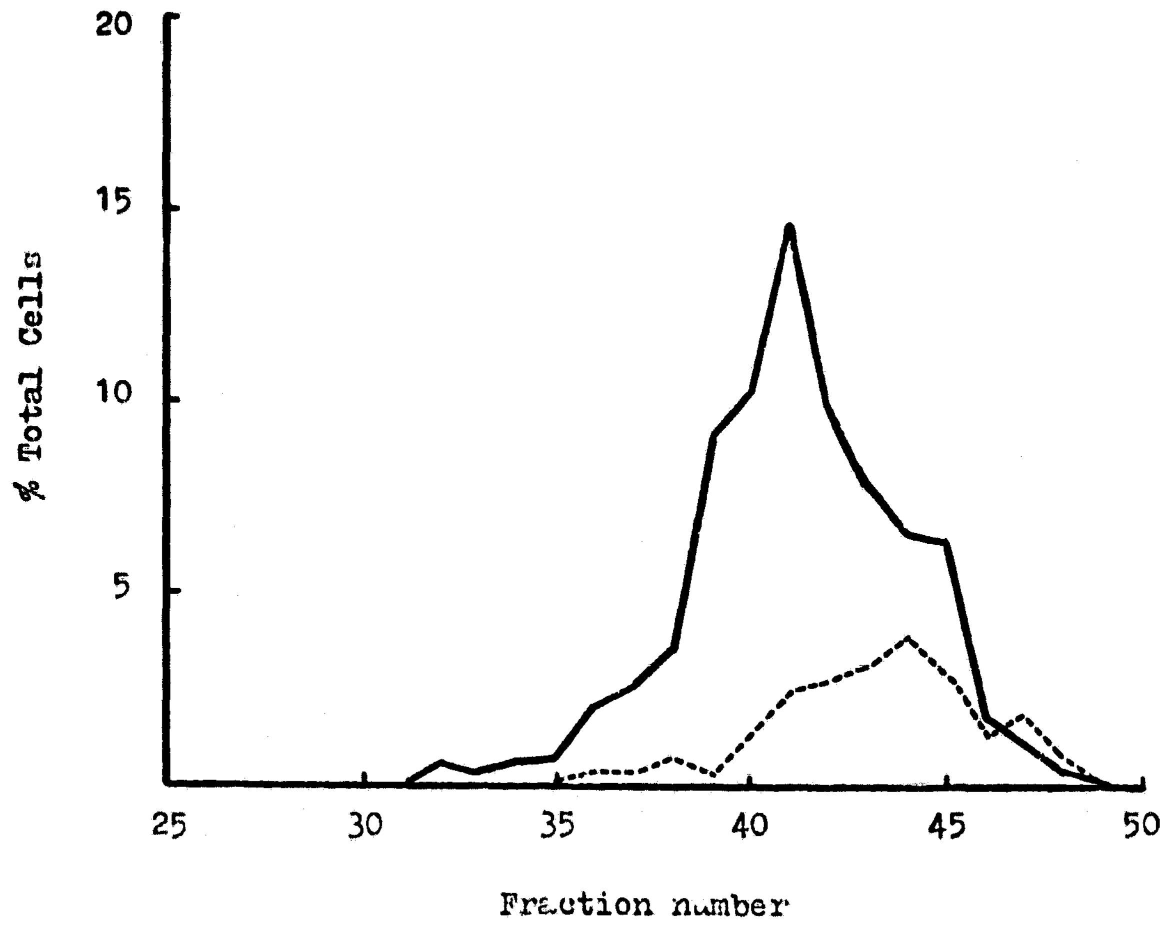

Figure 13. Free-flow electrophoretic separation of lymphocytes sensitized with rabbit anti-Smig then labelled with fluorescent PGL microspheres (----) from unlabelled lymphocytes (——). Acetate electrophoresis buffer flow rate = 10 ml/min.

free-flow electrophoresis experiments. Lymphocytes were sensitized with rabbit anti-Smig antibody and an aliquot of tha cells run on the FF 5 (solid line). The remaining cells were conjugated with fluorescent PGL microspheres showing an electrophoretic mobility of $-1.34 \mu sec^{-1}V^{-1}cm$, 0.2 units lower than microspheres previously used for lymphocyte labelling. Following free-flow electrophoresis, the distribution shown by the broken line in Figure 14 was obtained. Fractions 38 through 42 contained both fluorescent and non-fluorescent lymphocytes; fractions 43 through 48 contained fluorescent lymphocytes only, with few aggregated cells and viability in excess of 60% in all six fractions.

The results of free-flow electrophoresis of labelled and unlabelled lymphocytes showed the feasibility of enhanced electrophoretic separation using microspheres. The fact that increased resolution of surface immunoglobulin labelled lymphocytes was obtained with microspheres showing relatively slow electrophoretic mobility ($-1.34 \mu sec^{-1}V^{-1}cm$) suggests that higher resolution may be obtained using microspheres of even lower mobility. The slowest microsphere currently available is the HEMAC polymer DCM 253 with a mobility of $-1.20 \mu sec^{-1}V^{-1}cm$ (Table 5); this microsphere has yet to be tested in free-flow electrophoretic separations of lymphocytes. Further improvement in recovery of mono-disperse microsphere labelled lymphocytes may result from direct antibody labelling (Section 5.2.2).

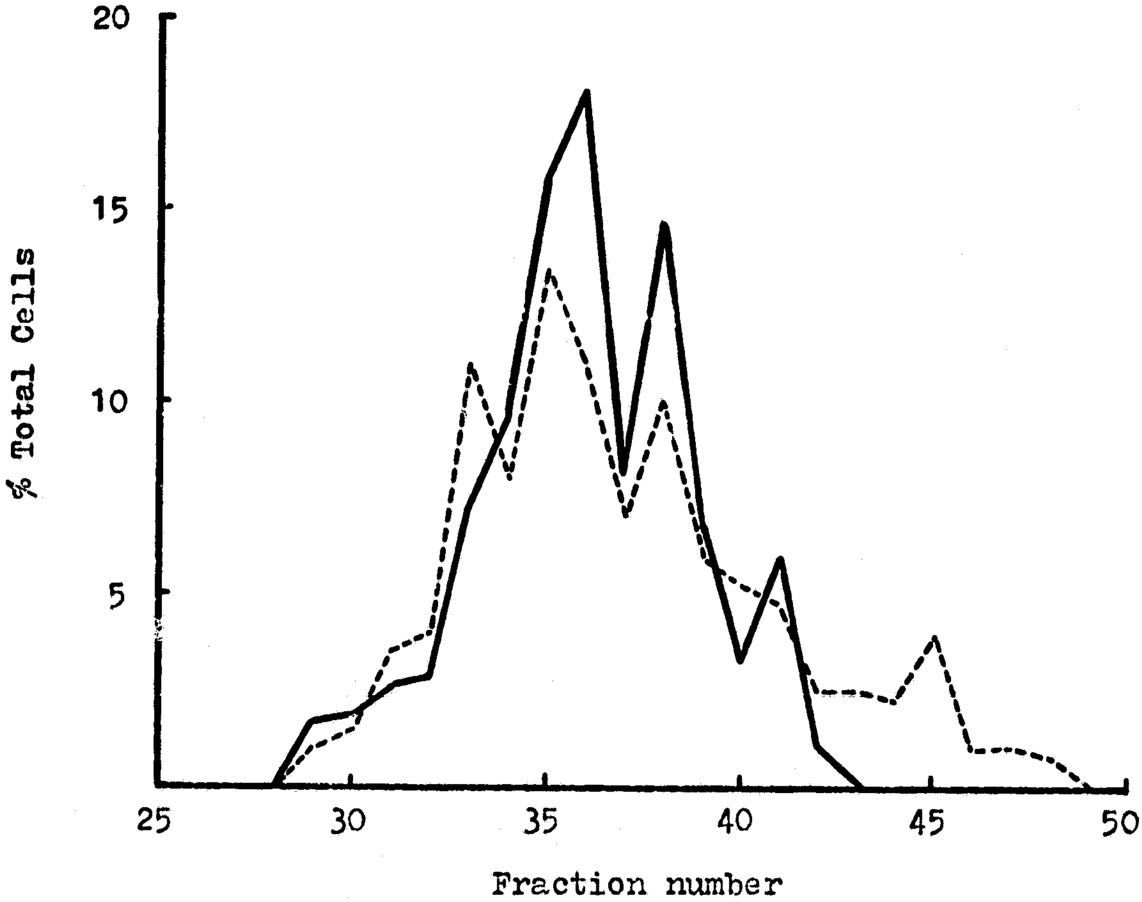

Figure 14. Free-flow electrophoretic distribution of lymphocytes sensitized with rabbit anti-Smig (——) and lymphocytes sensitized with rabbit anti-Smig then labelled with fluorescent PGL microspheres (-----).

6. Gastric Membrane Vesicles

6.1. Background

In addition to exploring the feasibility of lymphocyte separation using microspheres, an important objective of the contract studies is to identify other cell populations of interest to the biomedical community whose purification is difficult or impossible using current separation technology, and to apply the concept of microsphere-enhanced electrophoretic separation in such cases. A possible candidate cell population consists of the parietal cells of gastric epithelium. The function of the parietal cell is to produce hydrochloric acid, required in the stomach for the activation of pepsinogen by cleavage of a small peptide yielding the potent proteolytic enzyme pepsin. Secretion of protons into the secretory cannaliculus of the parietal cell is driven by adenosine triphosphate (ATP), and is mediated by a membrane bound ATP-ase incorporating selective ionic transport channels for K^+ and protons. The mechanism by which the chemical energy of ATP is converted into an electrochemical proton gradient of great steepness across the membrane of the secretory cannaliculus is of major current interest in membrane bioenergetics.

Fundamental to an understanding of proton secretory mechanisms in gastric epithelium is the aquisition of the purified ATP-ase with its associated ionic pumps. The most

highly purified vesicular $H^{+}K^{+}$ ATP-ase available today is prepared by differential centrifugation of hohogenized hog gastric mucosal scrapings, followed by zonal centrifugation of the microsomal fraction on sucrose-Ficoll gradients (18) Free-flow electrophoresis is used in the final purification step, which yields three fractions, one of which is greatly enriched in $H^{+}K^{+}$ ATP-ase activity. Significant levels of activity are still found, however, in the other two fractions. The concept of immunospecific microsphere enhancement of cell separation could improve the yield and specificity of the enzyme purification, simultaneously reducing the isolation procedure to a single step, free-flow electrophoresis of the mucosal homogenate, rather than the three stage method currently used.

6.1.2. Production of Monoclonal Antibodies

In order to effect a microsphere-based electrophoretic isolation of $H^{+}K^{+}$ ATP-ase-containing membrane vesicles, an antibody showing high specificity for the enzyme must be bound to appropriately charged microspheres with no loss of antigen specificity. Antibodies raised in the conventional manner against $H^{+}K^{+}$ ATP-ase by immunization of rabbits (28) or mice with purified vesicular preparations of the enzyme followed by bleeding show a wide spectrum of activities, even across species barriers, and in the case of the mouse, activity

against all three fractions of the free-flow electrophoretic final step of the H^+K^+ATP-ase isolation procedure (29) We have turned therefore to the production of monoclonal antibodies to the H^+K^+ ATP-ase by the technique of plasma cell hybridization (30-32).

Briefly, mice were immunized with purified hog H^+K^+ ATP-ase vesicles, their lymph nodes excised, and the lymphocytes fused in the presence of polyethylene glycol with a non-immunoglobulin secreting line of mouse plasmacytoma cells maintained in culture (33). After two weeks growth in selective medium which did not allow survival of unfused myeloma cells or lymphocytes, the culture supernatants of the fusion products were screened for activity against H^+K^+ ATP-ase vesicles by enzyme-linked immunoabsorbent assay. Several positive hybrid cell lines were identified; these were sub-cloned into fresh medium, and their supernatant activities tested again after 14 days continued growth. Ten hybrid clones showed positive reactions against the original antigen. Figure 15 these hybridoma antibody activities against three antigens, gastric epithelial fractions derived from hog, rabbit, and rat. Several of the antibodies show extensive cross-reactions, but three are specifically active only against the hog gastric mucosal H^+K^+ ATP-ase. The clones secreting these antibodies were sub-cloned again into fresh medium, and will subsequently be expanded first into 35 ml

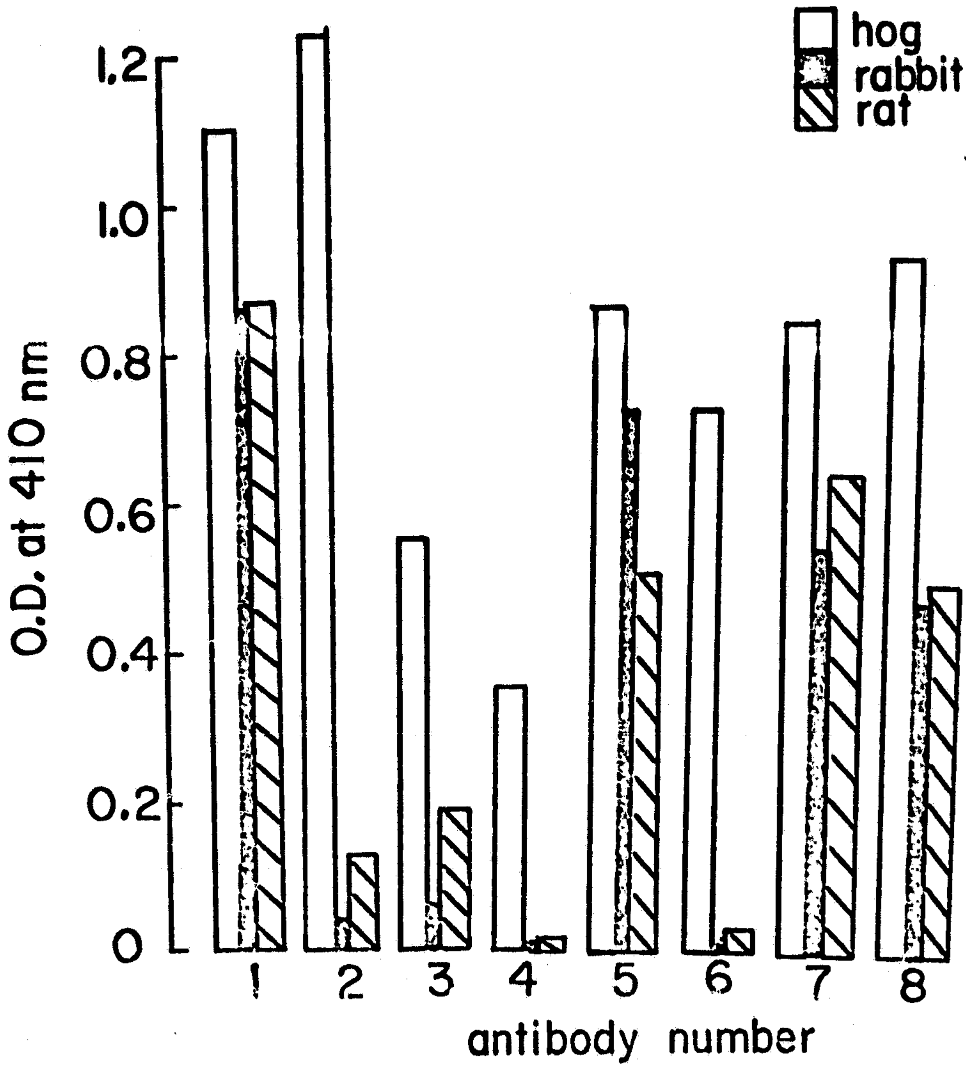

Figure 15. Activity of ten hybridoma-secreted antibodies against gastric epithelial membranes from hog, rabbit, and rat as measured by alkaline phosphatase-linked immunoabsorbent assay.

tissue culture flasks, and then injected as active secreting plasmacytomas into the peritoneal cavities of host mice for long term propagation.

This phase of research has already produced highly specific antibodies to the H^+K^+ ATP-ase of interest, and thus provides the basis for preliminary experiments to assess the applicability of the microsphere concept to improved purification of the enzyme. To this end, PGL microspheres will be incubated with selected monoclonal antibodies, and the derivatized microspheres then used to label a homogenate of the hog gastric mucosal scrapings. Free-flow electrophoresis of the homogenate may then allow quantitative recovery of those vesicles which incorporate the crucial H^+K^+ ATP-ase proton pump. These experiments will be actively pursued in the coming year.

7. Summary

PGL and HEMAC microspheres synthesized at the Jet Propulsion Laboratory were screened electrophoretically to determine their electrokinetic properties. These measurements were accomplished by calibration of a preparative free-flow electrophoresis apparatus; owing to the sub-microscopic dimensions of the microspheres, conventional cylindrical tube microelectrophoresis could not be used. The results of the microsphere studies showed minimal modification of mobil-

ity by covalently bound antibody, and maximal depression of mobility by post-synthetic quenching of microspheres in lysine.

Microelectrophoretic studies of SRBC confirmed the depression of cell electrophoretic mobilities by conjugation with PGL microspheres, and in addition revealed a small concomitant increase in cell isoelectric point as a result of microsphere binding. The latter result may be of significance in cell separation by isoelectric focusing.

Analogous microelectrophoretic studies of human lymphocytes correlated well with red cell analyses; lymphocyte mobilities were depressed, albeit to a lesser extent than SRBC, when microspheres were immunospecifically bound to the cell surface. Preparative free-flow separations showed a partial enrichment of labelled lymphocytes which correlated with the electrophoretic properties of the microspheres used for cell surface labelling. It is expected that as microspheres of lower mobility become available, the resolution of lymphocyte sub-group separation in free-flow electrophoresis will improve.

Finally, preliminary studies were initiated on the production of monoclonal antibodies to the H^+K^+ ATP-ase of hog gastric mucosa, with a view to utilization of these antibodies as coupling agents in the labelling of active gastric membrane vesicles with microspheres. Six monoclonal antibody-produc-

ing hybridomas are currently being grown to mass culture, and should prove useful in developing an improved purification of H^+K^+ ATP-ase based on microspheres. In addition, these monoclonal antibodies will be of great value as molecular probes in dissection of the structure and function of the various sub-units of the enzyme.

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